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14. ABSTRACT

We have shown previously that c-Src null epithelial cells are unable to respond to estrogenic stimulation [1]. In addition, c-Src recruitment to ErbB-2 catalytic domain could be involved in the hormone independent response of ErbB-2 induced mammary tumorigenesis [2]. In order to assess whether ablation of c-Src can influence the ability of mammary epithelial cells to respond to hormonal stimulation, we are currently generating a mouse strain homozygous for the loss of c-Src tyrosine kinase specifically in the mammary epithelium. We have also derived several independent mouse strains that express, under the control of an MMTV promoter, a chimeric EGF Receptor (TK) carrying the catalytic domain of ErbB-2 and thus able to recruit c-Src. Concomitantly, we have generated transgenic mice expressing wild-type EGFR under the transcriptional control of the MMTV promoter. Comparison of the mammary tumor incidence between these mouse strains will provide important insight into the importance of the recruitment of c-Src by ErbB-2 in hormone responsiveness of breast cancers.

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ERα, c-Src, breast cancer

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INTRODUCTION

The research objective outlined in the original proposal was to determine the significance of c-Src and ERα interaction in mammary tumor progression. This work was initially based on the observation that c-Src null epithelial cells are unable to respond to estrogenic stimulation [1] (see appended manuscript). We further demonstrated that c-Src recruitment to ErbB-2 catalytic domain could be a parameter in well known hormone independent response of ErbB-2 induced breast cancers [2] (see appended manuscript). The principle objective of the extension was to follow up on these key observations. Given that Src activity and protein levels are elevated in human tumor samples [3], this result may have important implications for understanding the molecular mechanisms of hormone resistant breast cancer.

BODY

1) Derivation of mammary specific knockout of c-Src tyrosine kinase.

The major goal outlined in the extension of this research proposal was to determine what the effect of mammary specific ablation of c-Src. One shortcoming of using the conventional germline is that it has major defects in estrogen signaling [1] thus making difficult to evaluate whether observed mammary epithelial defects are cell autonomous or cell non-autonomous in origin. To directly address this issue we have constructed a targeting vector where the critical first coding exon of c-Src is flanked by LOXP1 recombination sites (Figure 1).

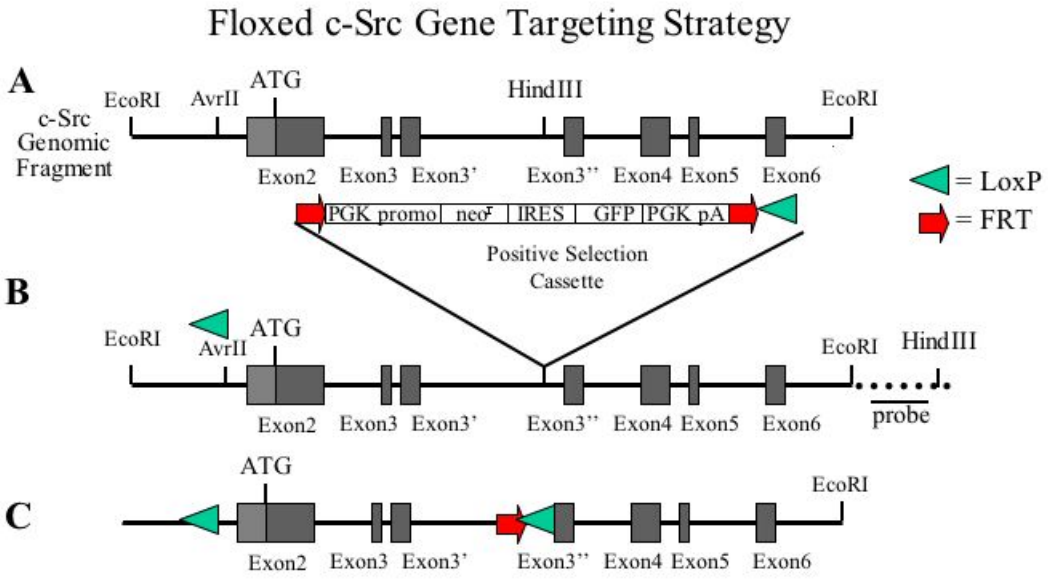

Figure 1;Strategy for generating floxed c-Src. A) Map of c-Src locus showing restriction site important for construction. B) Targeting construct with 5' loxP addition and FRT flanked postive selection cassette. Targeted ES clone were selected for Neo resistance and GFP expression. Positive clones were screened by PCR for integration of 5' loxP site and screened by Southern blotting using a 3' probe after Hind III digestion. C) Targeted locus after removal of the positive selection cassette.

We introduced this targeting vector into 129 ES cells and have derived two independent correctly targeted cell lines using this approach. After injection of these ES targeted clones into blastocysts, we have derived 10 chimeric mice. These mice are currently breeding and should result in germline transmission of this targeted c-Src allele. Once these mice have been successfully established, we will interbreed them with MMTV/Cre mice. The results of these crosses should allow us to assess whether mammary epithelial ablation of c-src can influence the ability of the mammary epithelial cell to respond to hormonal stimulation

2) Generation and characterization of transgenic mice expressing chimeric EGFR capable of binding c-Src.

A second major goal during the funding period was to determine if mammary specific expression of chimeric EGFR that has been engineered to bind c-Src would influence mammary tumor formation. To this end, we derived several independent strains that carry an EGFR which harbors the catalytic domain of ErbB-2(TK) (Figure 2) [2] under the transcriptional control of the MMTV promoter. In addition to TK receptor mice, we also derived transgenic mice expressing the wild type EGFR under the transcriptional control of the MMTV promoter. We are currently monitoring cohorts of 20 female mice from our best expressing lines for TK (233-2A2) and EGFR (266.6A3) for the development of tumors.

A

Construct	Founders	Expressors	Methods
EGFR	03-222-3	Not tested	
	03-223-1	positive	Real-Time PCR; IP/Western
	03-230-1	Not tested	
	03-230-5	Not tested	
	03-230-9	negative	Real-Time PCR
TK	03-206-5	positive	Real-Time PCR
	03-206-8	positive	Real-Time PCR
	03-206-9	positive	Real-Time PCR; IP/Western
	03-208-1	negative	Real-Time PCR; IP/Western
	03-220-1	positive	Real-Time PCR
	03-220-3	Not tested	

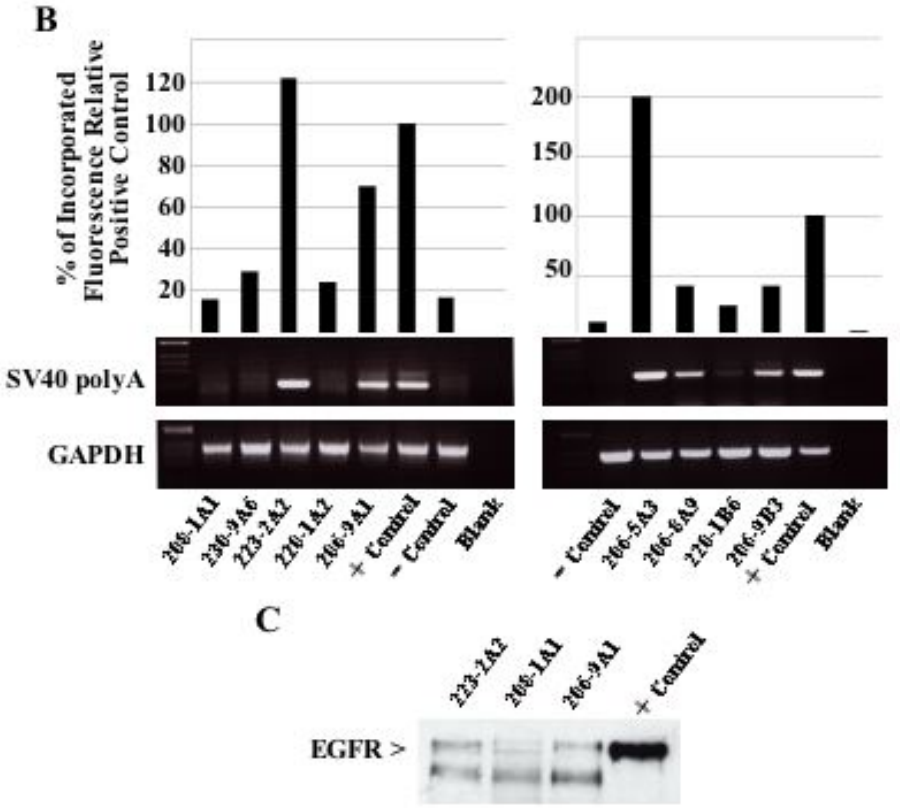

Figure 2 Legend: A) Summary of EGFR and TK transgenic mice. Five founders were positive for EGFR while six founder were positive for TK. Mammary glands of the offspring from these founders were tested for EGFR and TK expression. **B)** Total RNA extracted from the mammary glands of potential virgin EGFR and TK expressors were subjected to Real-Time PCR using primers targeted to the poly A tail of the MMTV construct. Expression is depicted relative to the positive control. GAPDH was used to confirm RNA integrity. **C)** Proteins extracted from the mammary glands of potential virgin EGFR and TK expressors were subjected to immunoprecipitation using an EGFR antibody. Immunoprecipitates were then processed by Western blot and probed with an EGFR antibody. Positive control was from a cell lysate transfected with a EGFR expression vector.

KEY RESEARCH ACCOMPLISHMENTS

- Generation and characterization of mice expressing TK and EGFR in the mammary epithelium, under transcriptional control of the MMTV-LTR
  Generation of chimeric mice bearing conditional targeted c-Src allele

REPORTABLE OUTCOMES

- Publications :
- Kim, H., Laing, M.A., **Muller, W.J.** (2005) The Estrogen Receptor and c-Src are Required for normal mammary gland development. Oncogene 24,5629-5636. (Appendix1)
- Kim, H., Chan R., Dankort, D.L., Zou, D, Naujokas, M., Park, M. and **Muller, W.J**. (2005). The c-Src tyrosine kinase associates with the catalytic domain of ErbB-2:implications for erbB-2 mediated transformation and signalling. Oncogene 24, 7599-760 (Appendix 2)

CONCLUSIONS

Funding for this project was provided in order to assess the role of c-Src in estrogen responsiveness. The most important and published result is the demonstration that the c-Src null animals have major defects in estrogen signaling [1]. Because of global defects in estrogen signaling observed in these c-Src deficient mice, we have recently generated mice that carry a conditional c-Src allele (Figure 1). With the availability of conditional c-Src strain, and MMTV/Cre transgenics [4] we will be able to directly assess whether mammary specific deletion of c-Src will impact on normal mammary gland development and estrogen responsiveness *in vivo*.

Another important advance was the generation of transgenic mice expressing an EGFR chimeric receptor that has be engineered to recruit c-Src. Based on our published observations that recruitment of c-Src by ErbB-2 may be an important step in hormone responsiveness, we would predict that mammary specific expression of the TK receptor will result in higher incidence of mammary tumors than EGFR alone. The results of these studies would suggest that c-Src-ERα and ErbB-2 are important therapeutic targets for treatment of breast cancer.

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APPENDICES (pages 9-25)

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ORIGINAL PAPER

The c-Src tyrosine kinase associates with the catalytic domain of ErbB-2: implications for ErbB-2 mediated signaling and transformation

Harold Kim[1,2], Richard Chan[3,4,5], David L Dankort[3,6], Dongmei Zuo[4,5], Monica Najoukas[4,5], Morag Park[4,5] and William J Muller*[,4,5,7]

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c-Src associates with and is activated by the ErbB-2 receptor tyrosine kinase, but is unable to bind the EGFR. Although c-Src has been found to interact directly and specifically with the ErbB-2 receptor, the significance of this interaction is unclear. Using both chimeric receptor and site-directed mutagenesis approaches, the region of interaction of c-Src on ErbB-2 was identified. Significantly, EGFR could be converted into a receptor capable of binding c-Src by replacement of a catalytic domain of ErbB-2. We further demonstrated that MDCK cells that express mutant EGFR that are competent in c-Src recruitment lose epithelial polarity in organoid cultures, whereas cells overexpressing the wild-type EGFR retain a polarized phenotype. ErbB-2-dependent activation of c-Src results in disruption of epithelial cell–cell contacts leading to cell dispersal that correlates with the re-localization of phospho-MAPK to focal adhesions. Taken together, these observations suggest that recruitment of c-Src to these closely related EGFR family members plays a critical role in modulating cell polarity.
Oncogene (2005) **0,** 000–000. doi:10.1038/sj.onc.1208898

Keywords: receptor; activation; kinase; transformation; tumorigenesis

Introduction

Many human malignancies are caused by the deregulation of growth factor receptors in conjunction with the cytoplasmic signaling molecules that associate with them. Indeed, studies have identified that approximately 30% of all breast cancer cases display an overexpression of the ErbB-2/Neu receptor that correlates with a poor clinical prognosis in both node positive and node negative women (Slamon *et al*., 1989; Andrulis *et al*., 1998). Moreover, between 40 and 60% of Ductal Carcinomas *In Situ* (DCIS) express high levels of c-ErbB-2 (Latta *et al*., 2002).

Direct evidence for the c-ErbB-2/Neu receptor tyrosine kinase (RTK) in transformation stems from numerous studies in cell culture as well as mouse models. For example, elevated expression of an activated version of *c-erbB-2/neu* in the mouse mammary gland driven by the murine mammary tumor virus (MMTV) promoter induces multifocal adenocarcinomas with rapid onset (Muller *et al*., 1988; Bouchard *et al*., 1989). The potent transforming activity of *erbB-2* in the mammary epithelium can be attributed to its capacity to associate with a number of key downstream signaling pathways. For example, in primary human breast cancer samples, at least 70% of the tyrosine kinase activity in the cytosolic fraction is due to c-Src (Ottenhoff-Kalff *et al*., 1992). Consistent with these human studies, we have demonstrated that tumors induced by the expression of activated ErbB-2 possess elevated c-Src activity (Muthuswamy *et al*., 1994). It has been further demonstrated that the increase in c-Src activity is due to the ability of activated ErbB-2 to form stable complexes with c-Src in an SH2-dependent manner (Luttrell *et al*., 1994; Muthuswamy and Muller, 1994, 1995b; Muthuswamy *et al*., 1994; Belsches-Jablonski *et al*., 2001). Another striking feature of c-Src association with the EGFR family was that it was restricted to the ErbB-2 receptor and could not directly interact with the closely related EGFR (Muthuswamy and Muller, 1995a, b). Taken together, these observations argue that c-Src plays a critical role in ErbB-2 mediated transformation. Although the evidence suggests the importance of c-Src in EGFR family mediated mitogenesis and transformation, the specific molecular mechanism of c-Src activation and recruitment is unclear.

To identify the region of interaction of c-Src with ErbB-2, we have employed both chimeric receptor and a site-specific mutagenesis approaches. The results revealed that c-Src associates within the catalytic domain

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of ErbB-2. We further demonstrated that epithelial cells that express a chimeric EGFR receptor engineered to associate with and activate c-Src lose cell polarity and cell–cell junctions and disperse in response to EGF. Finally we show that the c-Src-dependent dispersal response of epithelial cells is correlated with the relocalization of phospho-MAP kinase to the cytoplasmic membrane. These observations suggest that the ability of ErbB-2 to specifically recruit c-Src results in the breakdown of cell–cell adhesions and dispersal of organized epithelium that could contribute to metastatic invasion.

Results

The carboxyl terminal region does not mediate the association of Src to the ErbB-2 RTK

In order to address systematically whether the major autophosphorylation sites on ErbB-2 mediate the association of c-Src to the ErbB-2 RTK, we assessed the ability of c-Src to associate with a series of activated ErbB-2 (NeuNT; Bargmann *et al*., 1986; Bargmann and Weinberg, 1988) mutants harboring individual mutations in each of the known tyrosine autophosphorylation sites (Figure 1a). Direct binding assays using an Src-SH2 GST fusion protein revealed that the individual removal of any of the five autophosphorylation sites from the NeuNT receptor resulted in the association of the c-Src SH2 domain to the receptor, similar to the association observed with NeuNT (Figure 1b, lanes 1–7) in a tyrosine phosphorylation-dependent manner (Figure 1c, lanes 3–7). These results suggest that the alteration of any single tyrosine residue tested in the carboxyl terminal region does not affect the association of c-Src to the receptor and that potentially other tyrosine residues may mediate the association with c-Src.

In order to identify whether any of the five major tyrosine autophosphorylation sites can independently mediate the association of c-Src to the NeuNT receptor, mutant ErbB-2 receptors bearing single tyrosine autophosphorylation sites (add-back mutants) were subjected to identical direct binding analysis (Figure 1b, lanes 1, 2, 9–13). The results revealed that all tested ErbB-2 add-back mutants were capable of directly binding the c-Src SH2 domain (Figure 1c, lanes 9–13). Significantly, ErbB-2 mutants lacking all of the known tyrosine phosphorylation sites (NT-NYPD) still retained the capacity to bind to the c-Src SH2 domain (Figure 1b, lane 8). Indeed, despite the loss of five major tyrosine phosphorylation sites, the NYPD mutant was efficiently tyrosine phosphorylated (Figure 1c, lane 8). To confirm the *in vitro* binding studies, we also tested whether the NYPD mutant could be co-immunoprecipitated with c-Src. Consistent with the direct binding analyses, c-Src could efficiently associate with the NYPD mutant *in vivo* (Figure 1d, lane 3). Taken together, these observations suggest that c-Src interacts with a tyrosine residue on

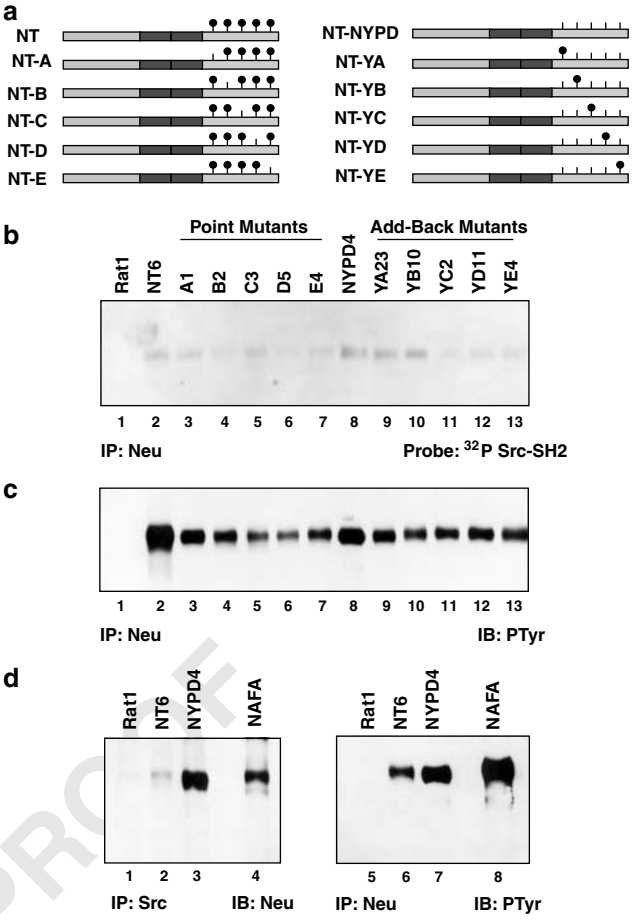

Figure 1 c-Src does not associate with the five-autophosphorylation sites on NeuNT. (**a**) Schematic representation of the NeuNT receptor (NT). Dark bars represent the kinase region, and light bars represent nontyrosine kinase domains. Various constructs as point-mutants that remove each autophosphorylation site (black circles) and replace it with a phenylalanine residue (NT-A to E) or NT with all the autophosphorylation sites removed (NT-NYPD) or the add-back mutants where each tyrosine is replaced in the context of NYPD (NT-YA to YE) are shown. (**b**) Each NT mutant was immunoprecipitated and the ability of a GSTagSrcSH2 fusion protein to associate directly to each receptor was assessed. (**c**) Neu was immunoprecipitated and subjected to an antiphosphotyrosine immunoblot. (**d**) The ability of c-Src to interact with NeuNT (NT6), NYPD or a mammary cell line that expresses high levels of NeuNT (NAFA) were analysed via *in vivo* co-immunoprecipitation

ErbB-2 that is distinct from the five major autophosphorylation sites.

The catalytic domain mediates the association of ErbB-2 with c-Src

Previous studies suggested that unlike ErbB-2, c-Src is unable to directly interact with the closely related EGFR (Muthuswamy and Muller, 1995b). Given the differential binding observed between c-Src and the members of the EGFR family, we took advantage of chimeric receptors between EGFR and ErbB-2 (Figure 2a) to define the region on ErbB-2 necessary for c-Src association. To this end, co-immunoprecipitation ana-

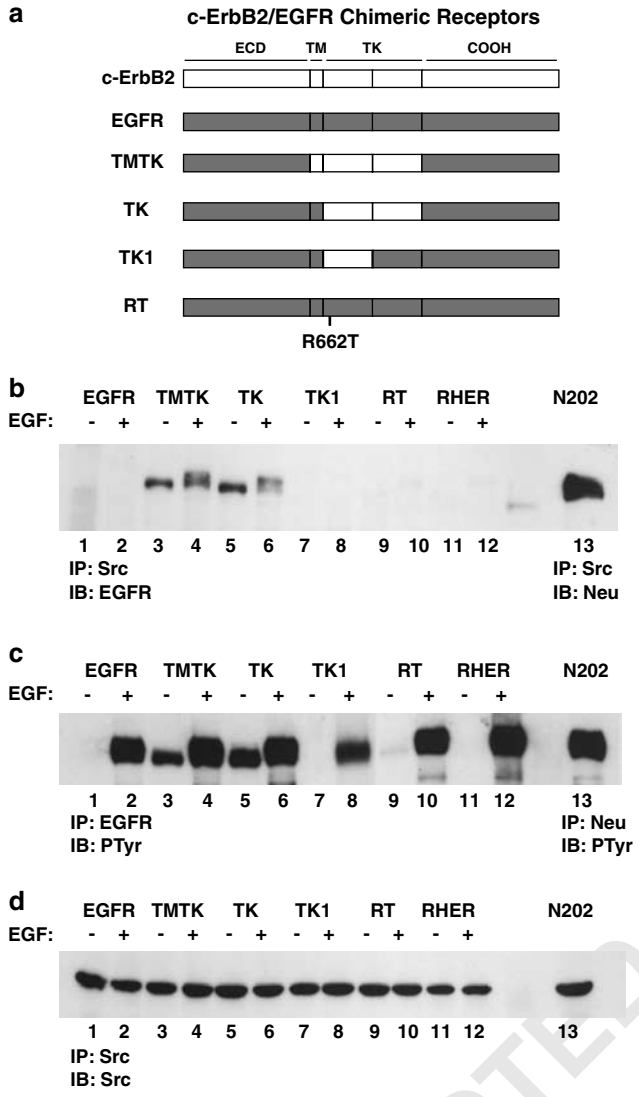

Figure 2 c-Src associates specifically with TMTK and TK. Co-immunoprecipitation analysis of stable cell lines expressing the various chimeric constructs. (**a**) The structures of the EGFR/c-ErbB-2 chimerics are shown, dark bars representing the EGFR, and light bars representing the ErbB-2 receptor. (**b**) c-Src was immunoprecipitated with anti-Src (7D10) from stable cell lines expressing the various chimeric constructs and the presence of the EGFR within the immunocomplex was assessed both in the absence and presence of 100 ng/ml EGF for 5 min at room temperature. (**c**) The presence of tyrosine phosphorylation upon EGF stimulation on each receptor was analysed by immunoprecipitating the chimeric receptors and immunoblotting with antiphosphotyrosine (PY20). (**d**) The levels of c-Src in each immunoprecipitation were analysed by immunoblotting with anti-Src (Ab1). Protein lysate from the ErbB2 induced mouse tumor model (N202) was used as a positive control

lysis between c-Src and the chimeric receptors demonstrate that upon EGF stimulation, an increase in c-Src association was observed in two chimeric receptors that harbor an intact ErbB-2 kinase domain, TMTK and TK (Figure 2b, lanes 3–6). Interestingly, the chimeric receptor TK1 that is missing the carboxyl terminal region of the ErbB2 kinase domain does not appear to be able to associate with c-Src (Figure 2b, lanes 7 and 8). While the above data suggests that c-Src physically maps to the catalytic region of the receptor, it may be possible that the kinase activity dictates the specificity of c-Src to the EGFR/ErbB2 chimera by differential phosphorylation of the receptor. To address this, we tested the ability of c-Src to associate with a mutant EGFR (RT) that harbors a single point mutation within its juxtamembrane region that confers an ErbB2-like phenotype in the context of an EGFR kinase domain (Di Fiore *et al*., 1990). In contrast to the chimeric receptors harboring the ErbB-2 catalytic domain, the RT mutant failed to associate with c-Src (Figure 2b, lanes 9 and 10). Given the above data, this suggests that c-Src associates specifically with the kinase domain of ErbB2.

Recruitment of c-Src by the chimeric EGFR disrupts epithelial cell polarity and promotes epithelial cell dispersal

To assess whether the observed capacity of ErbB-2 to recruit c-Src influences epithelial cell polarity and dispersal, we established several independent MDCK cell lines expressing activated c-Src, EGFR or the TK chimeric receptor. Immunoblot analyses with several independent clones of MDCK cells expressing different chimeric receptors revealed that they expressed comparable levels of activated RTKs following stimulation with EGF (Figure 3d). To ascertain whether recruitment of c-Src influenced the capacity of these cells to form organized epithelial structures, we examined the behavior of the different MDCK clones in collagen gels following EGF stimulation. Initial comparisons of EGFR and TK expressing clones revealed that in the absence of EGF, the majority of the clones when seeded in collagen gels formed cystic structures that comprise of a single layer of polarized epithelial cells (Figure 3a). Upon EGF stimulation, the majority of the EGFR expressing cells retained these cystic structures (Figure 3a, top panels). In contrast to the EGFR expressing cells, the TK expressing MDCK cells exhibited a dramatic scattering and invasion phenotype in response to EGF (Figure 3a, bottom panels). Higher magnification of these structures revealed that the TK receptor expressing cells exhibited disorganized and dispersed structures that closely resembled MDCK cells that stably expressed an activated c-Src kinase (Figure 3b, bottom panels). Quantitative analyses of multiple independent clones confirmed that the majority of MDCK cell lines expressing the TK mutant exhibited a dramatic migratory response following EGF stimulation (Figure 3c). Significantly, a high proportion of MDCK cells expressing activated c-Src exhibited a dispersed phenotype following EGF stimulation. In contrast, only a small proportion of MDCK cells expressing the parental EGFR exhibited a dispersed phenotype in response to EGF stimulation (Figure 3c).

Since expression of activated ErbB-2 in MDCK cells has been previously implicated in the induction of epithelial-to-mesenchymal transition (EMT) (Khoury *et al*., 2001), we next determined whether the migratory

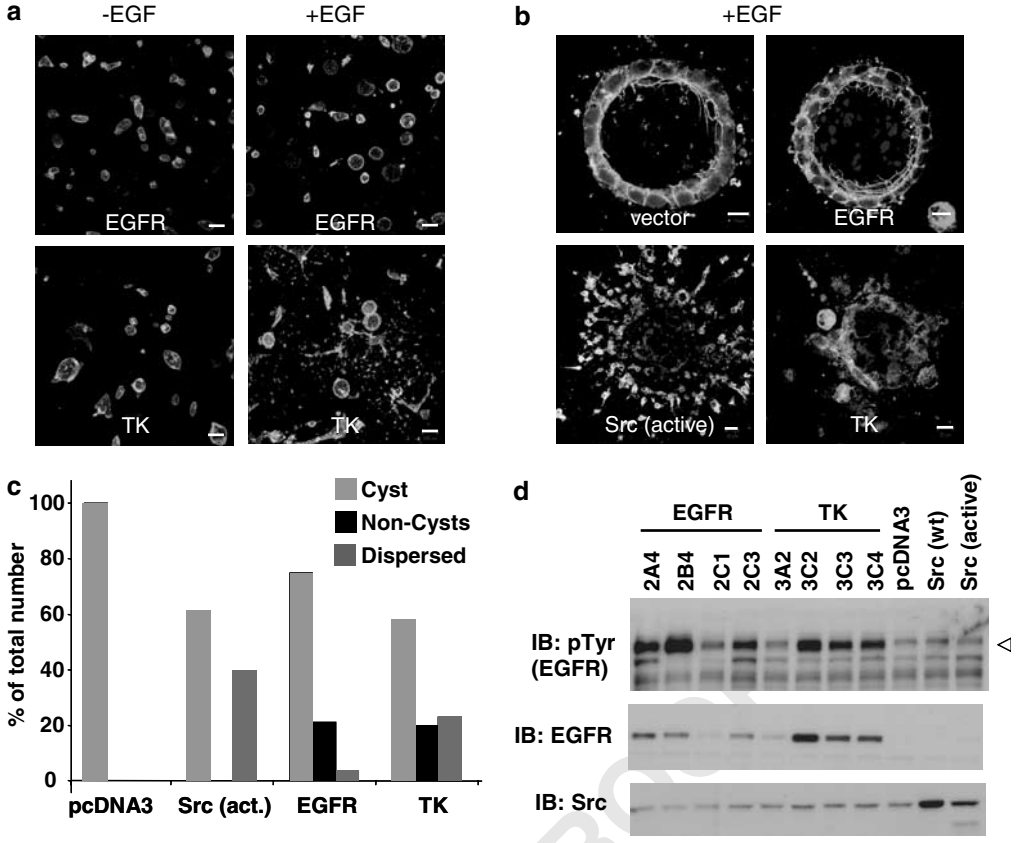

Figure 3 Activation of the TK mutant disrupts MDCK cystic structures in collagen. Stable lines of MDCK cells expressing either the EGFR or TK receptor were grown in a matrix of type I collagen (Vitrogen 100 – Cohesion Technologies). After 4–6 days, cultures were stimulated with EGF (100 ng/ml). Medium was renewed every 5 days and cultures were fixed after 2 weeks. (**a**) Overview of the MDCK cystic structures formed in the collagen assay. Scale bar = 100 μm. (**b**) Detailed view at higher magnification of individual MDCK three-dimensional structures formed in the presence of EGF stimulation. MDCK cells expressing the empty vector (pcDNA3) and an active Src PTK were included for comparison with the EGFR and TK expressing clones. Scale bar = 10 μm (top panels) and 20 μm (bottom panels). (**c**) Enumeration of the structures formed in the collagen assays. The results are a compilation of multiple MDCK clones expressing the indicated plasmids and counted over at least three fields. (**d**) Immunoblot comparing the levels of EGFR, phosphotyrosine and Src in the indicated MDCK stable clones that were used in the collagen assays

response of these cells expressing different chimeric EGFR was related to a loss of epithelial cell polarity. To accomplish this, MDCK cells expressing EGFR or the TK mutant were assessed for the presence of the adherens junctional protein, E-cadherin, or the tight junction protein, ZO-1 (Figure 4a). Examination of MDCK cells expressing EGFR revealed that these cells retained a polarized phenotype in either the absence or presence of EGF, while stimulation of MDCK cells expressing the TK chimeric receptor exhibited the loss of both tight junctions and adherence junctions as determined by the intense cytoplasmic staining of both ZO-1 and E-cadherin (Figure 4a). These observations suggest that an EGF dependent migratory response exhibited by the MDCK cells expressing the TK chimeric receptor is associated with the breakdown of these important cell junctional complexes. Significantly, the use of the c-Src inhibitor PP2 on cell lines expressing the TK mutant rescue the breakdown of both the adherens junctional protein, E-cadherin and the tight junction protein, ZO-1 in the presence of EGF (Figure 4b), suggesting the importance of c-Src in ErbB2 mediated EMT response.

Recruitment of c-Src by the chimeric EGFR receptor results in the relocalization of activated MAP kinase to focal adhesions

Given the dramatic impact on the migratory behavior of MDCK cells expressing the TK chimeric receptor, we next examined whether the cellular localization of downstream components of the EGFR signaling cascade were altered as a consequence of the capacity to recruit c-Src. To test this possibility, we assessed both the levels and localization of phosphorylated MAPK in stable MDCK clones expressing the different chimeric EGFR receptors following stimulation with EGF. Measurement of the levels of phosphorylated MAPK after stimulation with the various MDCK clones showed that in clones expressing either the EGFR or TK receptor, phosphorylation of MAPK peaked at 5 min poststimulation (Figure 5a). The increase in the levels of MAPK phosphorylation was not due to differences in the level of MAPK as all clones expressed comparable levels of MAPK. When assessing the general kinetics of c-Src activation upon EGF stimula-

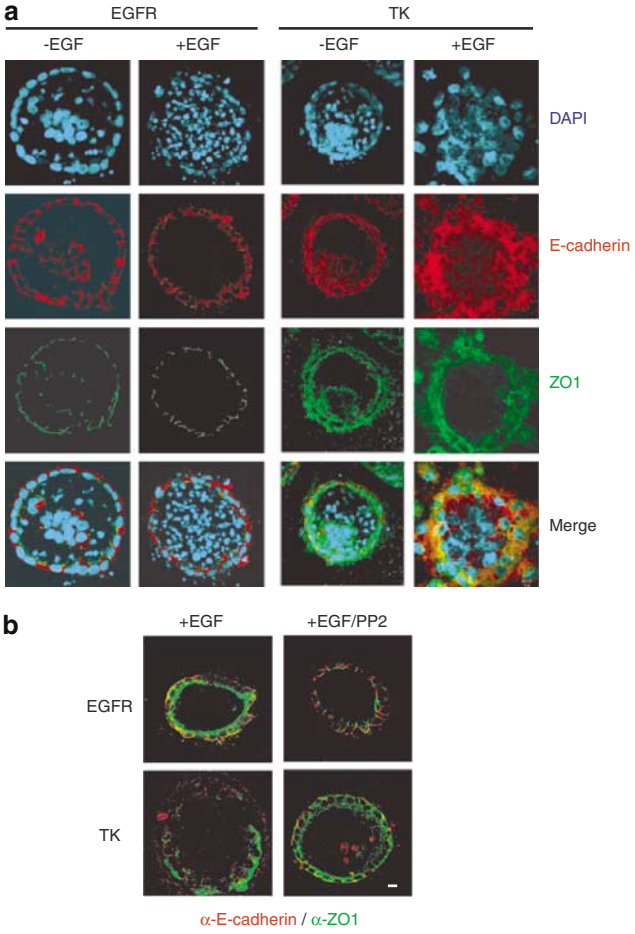

Figure 4 Expression of cell junction proteins in MDCK cysts grown in collagen. (**a**) Following the growth of EGFR or TK expressing MDCK stable clones in the absence or presence of EGF stimulation in collagen assays, the cystic structures were immunostained using anti-E-cadherin (red) and anti-ZO-1 (green) antibodies. Cell nuclei were stained with DAPI. Detailed confocal image of a representative structure is shown for each clone. (**b**) Growth of EGFR or TK expressing MDCK stable clones was treated with either EGF alone or together with EGF and PP2. Structures were immunostained using anti-E-cadherin (red) and anti-ZO-1 (green). Scale bar = 10 μm

tion, it was found that EGFR stimulation results in modest c-Src activation; however, the activation profile of c-Src when in association with TK appears more robust at all timepoints measured relative to EGFR overexpressing cells (Figure 5b).

Although these biochemical studies suggested that the kinetics of MAPK activation were comparable in MDCK cells expressing the EGFR or the TK receptor, staining of these cells with antibodies specific to phospho-MAPK revealed a dramatically different cellular localization in MDCK cells expressing the chimeric receptor. In both the parental MDCK and EGFR expressing MDCK cells, phospho-MAPK was initially detected primarily in the nucleus (Figure 5c). Following 5 min of stimulation, an increase in nuclear localization of phospho-MAPK was noted in both parental and EGFR expressing MDCK cells. In contrast to these results, MDCK cells expressing the TK chimeric receptors possessed the bulk of phospho-MAPK staining at points of focal adhesions at the tips of actin stress fibers in both resting and stimulated cells (Figure 5c). Thus, similar to rat fibroblasts expressing activated c-Src (Fincham *et al*., 2000), the chimeric EGFR that is competent for binding c-Src re-localizes phospho-MAPK to focal adhesions.

Given the above data, it is conceivable that MAPK activity is required for induction of migratory phenotype displayed by the MDCK cells expressing the TK chimeric receptor. To explore this possibility, we assessed whether the potent MEK inhibitor (U0126) could interfere with the capacity of the TK chimera to disrupt cell polarity. Following stimulation with EGF in the presence or absence of U0126, EGF stimulation of MDCK or EGFR expressing MDCK cells did not disrupt these cell junction complexes (Figure 6). Additionally, EGF stimulated cells expressing the TK chimera exhibited a dramatic disruption of these cell junction complexes. Remarkably, administration of the U0126 inhibitor completely prevented this disrupted cell architecture (Figure 6). Taken together, these observations argue that the localization and activation of MAPK may be required for the TK receptor to disrupt epithelial cell polarity in response to EGF stimulation.

Discussion

Although previous studies have implicated c-Src as an important mediator of ErbB-2 induced tumorigenesis, the precise mechanism by which this occurred remains to be elucidated. Employing a site-directed mutagenesis and chimeric receptor approach, we demonstrate that c-Src does not associate with any of the phosphotyrosine residues found within the tail of the ErbB-2 RTK but does associate with the carboxyl terminal region of the ErbB-2 catalytic domain. We further demonstrated that recruitment of c-Src to the ErbB-2 catalytic domain resulted in disruption of epithelial cell polarity that is dependent on the re-localization and activation of MAP kinase at focal adhesions. Given the importance of a loss of epithelial cell polarity in metastatic disease processes (Tapon, 2003), these observations provide a potential molecular explanation for the ability of ErbB-2 to induce metastatic epithelial tumors.

Interestingly, the importance of c-Src association to the catalytic region of an RTK has been previously reported. Studies have shown that c-Src can phosphorylate and associate, albeit weakly, with tyrosine residues in the catalytic domain of the PDGFβ receptor (Kypta *et al*., 1990; Hansen *et al*., 1996). Furthermore, the elevated expression of c-Src results in the hyperphosphorylation of tyrosine residues that includes the activation loop of the EGFR (Biscardi *et al*., 1999), specifically Y845 within the kinase region (Stover *et al*., 1995). Within the kinase domain, the activation loop has been found to play an important role in receptor function, acting much like a molecular gate that permits

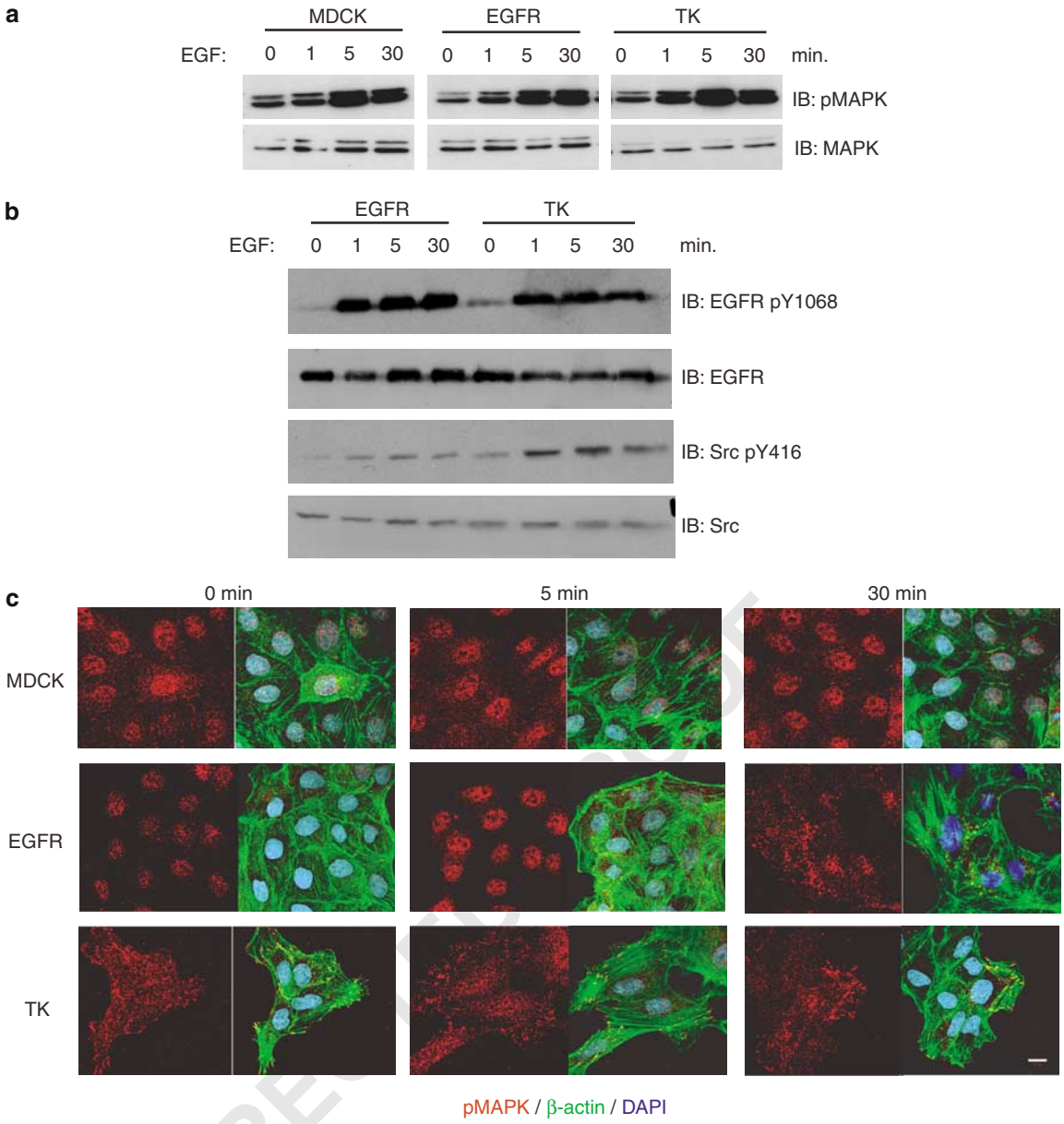

Figure 5 MAP kinase expression in MDCK clones grown in 2D cultures. (**a**) MDCK stable cells were serum-starved overnight then stimulated by the addition of 100 ng/ml of EGF for the indicated times. Phospho-MAPK and total MAPK levels were determined by immunoblot analyses of cell lysates derived from these cultures (Cell Signaling Technology). (**b**) COS7 cells transiently transfected with either the EGFR or the mutant TK construct were serum starved overnight and stimulated with 100 ng/ml of EGF for the indicated times. Phospho-EGFR, total EGFR, phospho-Src and total Src levels were determined by immunoblot analysis. (**c**) To track the localization of MAPK proteins in the MDCK cells, parallel cultures of stimulated MDCK cells were also immunostained for phospho-p44/42 (pMAPK; red) and actin cytoskeleton (green). Cell nuclei were counterstained with DAPI. Scale bar = 10 μm

ATP to enter the binding pocket. Indeed, the activation loop of the insulin receptor harbors three tyrosine residues that are important for the stabilization of the activation loop leading to ATP binding, substrate phosphorylation and full receptor activation (Hubbard *et al*., 1998). Interestingly, the region of the kinase domain of ErbB-2 that correlates with c-Src association, referred to as TK2 (Segatto *et al*., 1991), harbors the activation loop at tyrosine 882. Significantly, mutation of this residue on ErbB-2 results in the impairment of catalytic activity (Zhang *et al*., 1998); however, mutation of the same tyrosine on EGFR has no significant effect on EGFR activity (Gotoh *et al*., 1992; Tice *et al*., 1999). It is possible that following receptor dimerization, c-Src phosphorylates tyrosine 882 and creates a binding site for c-Src. Once c-Src associates with the catalytic domain it can transphosphorylate other EGFR family members within the catalytic domain and thus potentiate receptor activation. While previous data in conjunction with our data suggest that both c-ErbB2 and the

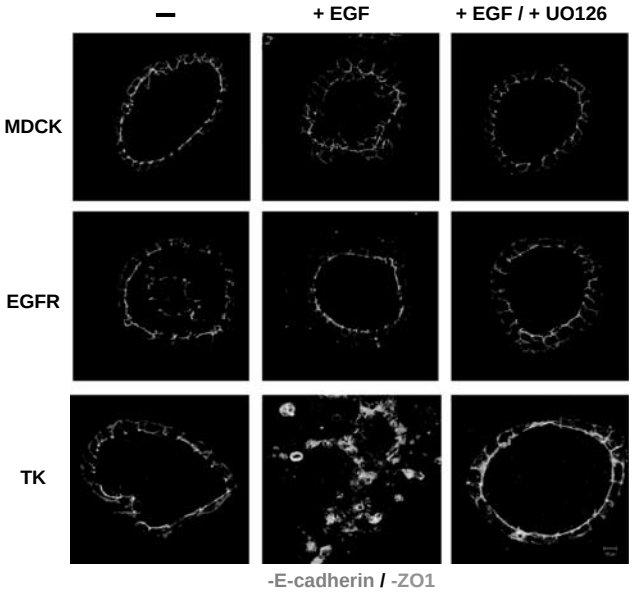

Figure 6 MAPK inhibitor prevents TK-induced MDCK scattering. MDCK cell lines expressing either the EGFR or TK receptors were grown in a matrix of type I collagen. After 4–6 days, EGF (100 ng/ml) alone or EGF and the specific MAPK inhibitor, U0126 (5 μM) were added. Growth medium was changed every 5 days. The assay was fixed after 2 weeks and cystic structures were immunostained with anti-E-cadherin (red) and anti-ZO-1 (green) antibodies. Detailed views of representative structures formed by the different clones under the indicated conditions are shown

EGFR can associate with c-Src, our data also suggest that c-ErbB2 appears to mediate the primary association of c-Src to this receptor family.

This model of c-Src mediated RTK activation may have important implications in the capacity of ErbB-2 to serve as the central player in EGFR family signaling (Karunagaran *et al*., 1995). The observation that c-Src augments the mitogenic response of cells to EGF (Luttrell *et al*., 1988; Maa *et al*., 1995; Belsches *et al*., 1997; Tice *et al*., 1999) is consistent with the idea that recruitment of c-Src to ErbB-2/EGFR heterodimers may be a critical step in EGFR transphosphorylation. Another EGFR family member that may be dependent on the ErbB-2/c-Src complex is ErbB-3. In contrast to other EGFR family members, ErbB-3 is naturally catalytically inactive (Guy *et al*., 1994) and is thus dependent on transphosphorylation by other EGFR family members. As RTK activation is thought to be dependent on the juxtaposition of two competent tyrosine kinase catalytic domains, it is conceivable that the ErbB-2/c-Src complex may serve as an active kinase involved in ErbB-3 transphosphorylation. Indeed, mammary tumors expressing activated ErbB-2 possess high levels of tyrosine phosphorylated ErbB-3 (Siegel *et al*., 1999).

Another important finding of our studies is that recruitment of c-Src to the ErbB-2 catalytic domain in the chimeric EGFR mutant (TK receptor) promotes the loss of epithelial polarity in three-dimensional organoid cultures. Notably, the stimulation of MDCK cells expressing the TK receptor resulted in the relocation of E-cadherin and ZO-1 cell junctional proteins to a cytoplasmic compartment, whereas the stimulation of cells expressing EGFR failed to promote the breakdown of cell–cell junctions (Figure 4a). We further demonstrate that this dramatic disruption of epithelial cell polarity is correlated with the re-localization of phospho-MAP kinase to focal adhesions and is dependent on a functional MEK/MAP kinase cascade (Figure 5c). Consistent with these observations, previous studies with MDCK or MCF-10A cells engineered to express activated forms of ErbB-2 exhibited alterations in their epithelial morphology (Khoury *et al*., 2001; Muthuswamy *et al*., 2001). Furthermore, activation of ErbB-2 but not the EGFR, in MCF-10A cells, exhibit luminal filling (Muthuswamy *et al*., 2001); however, in contrast to our observations epithelial adherens and tight junctional proteins were maintained (Muthuswamy *et al*., 2001). The difference in the phenotypic response to ErbB-2 in the previous study may be due to the regulated generation of ErbB-2 homodimers and subsequent activation (Muthuswamy *et al*., 2001), whereas the chimeric EGFR/ErbB-2 receptor is capable of forming heterodimers with other members of the EGFR family. This further suggests that epithelial cell polarity involves the cooperation between ErbB-2 and c-Src signaling and the constellation of signaling molecules coupled to the carboxy-terminus of the EGFR.

The importance of c-Src recruitment to RTKs initiating the disruption of epithelial cell polarity has recently been described for MCF-10A cells expressing activated forms of CSF-1R (Wrobel *et al*., 2004). In a similar manner to MDCK cells expressing the TK chimeric receptor, autocrine activation of CSF-1R results in the dramatic relocalization of cell junction proteins to a cytoplasmic compartment that is dependent on the recruitment of c-Src to the activated CSF-1 receptor (Wrobel *et al*., 2004). Indeed, EGF stimulation of MDCK cells expressing the TK mutant results in c-Src phosphorylation on tyrosine 416, cell dispersion and the disruption of epithelial cell polarity. Significantly, the inhibition of c-Src in MDCK/TK mutant cells rescues the dispersed phenotype and epithelial cell polarity (Figure 4b). In addition to the recruitment of c-Src to the TK chimeric receptor, a dramatic re-localization of phospho-MAPK occurs as early as 5 min post-EGF stimulation, from the nuclear compartment to sites of focal adhesion. While, EGF stimulation of both the EGFR and TK/MDCK cells results in a similar phospho-MAPK pattern by 30 min (Figure 5c), over time a clear difference in morphology develops, that is, TK are dramatically dispersed while EGFR cells retain their parental morphology (Figures 3 and 4a). Given our biochemical data implicating c-Src in this process in addition to the demonstration that disruption of epithelial cell polarity is dependent on MEK/MAPK signaling, we suggest that c-Src plays an important role in epithelial cell polarity and dispersion mediated by c-ErbB2. Consistent with these results, it has previously been reported that fibroblast cell lines expressing v-Src have re-localized phospho-MAPK to newly formed focal adhesions (Fincham *et al*., 2000). More recently,

it has been demonstrated that localized c-Src signaling through MAPK regulates adhesion disassembly that is critical in modulating cell migration (Webb *et al*., 2004). Given the demonstrated ability of ErbB-2 to promote the migratory behavior of metastatic breast cancer tumor cells, it is conceivable that ErbB-2 mediated re-localization of c-Src and MAPK plays a critical role in modulating the migratory behavior of tumor cells. Future studies with tissue specific ablation of c-Src in these ErbB-2 mammary tumor models should allow these issues to be addressed.

Materials and methods

PCR mutagenesis and DNA constructs

The NeuNT (V664E) cDNA was provided by R Weinberg. The wild-type EGFR and the TMTK, TK, TK1 and RT cDNA constructs were provided by PP Di Fiore. The EGFR and TK cDNA were subsequently cloned into the pcDNA3 expression vector and used to generate MDCK stable cell lines. All autophosphorylation point mutations generated in the context of the NeuNT cDNA and were described previously (Dankort *et al*., 1997). All nucleotide primers and sequencing were performed by the MOBIX Central Facility of McMaster University. The phosphoglyceraldehyde kinase-Puromycin plasmid (PGK-Puro) was obtained from MA Rudnicki. The c-SrcSH2 protein was a gift from B Margolis.

Cell lines, transformation assays and collagen assays

Rat1 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum with penicillin, streptomycin and amphotercin B. Stable Rat1 cell lines were established as previously described (Dankort *et al*., 1997). RHER (R1/HER) represents a stable fibroblast cell line that overexpresses the EGFR, while the NAFA cell line is a mammary epithelial cell that overexpresses NeuNT. Both have been described previously (Muthuswamy and Muller, 1995b). COS7 cells were transiently transfected with either the EGFR or TK mutant constructs as per the manufacturers instructions (Lipofectamine, Invitrogen). Subsequent to serum starvation, cells were stimulated with 100 ng/ml EGF for the required times.

MDCK cell collagen assays were performed as previously described (Khoury *et al*., 2001). Briefly, MDCK cell lines, seeded at a concentration of 10^4 cells/17 mm well, were allowed to form three-dimensional structures for 4–6 days whereupon EGF alone or with inhibitor was added. Medium was changed and U0126 inhibitor (Promega) renewed every 5 days. The assay was fixed and stained after approximately 2 weeks. (Khoury *et al*., 2001).

Fluorescent immunostaining and microscopy

Cells were fixed and immunostained following standard procedures. The secondary antibodies, goat anti-mouse (Alexa555 or Alexa488) and goat anti-rabbit (Alexa555 or Alexa488) were purchased from Molecular Probes. All cell images were taken using the Zeiss LSM 510 laser scanning confocal microscope.

Immunoprecipitation, immunoblotting and direct binding assays

Immunoprecipitation analyses were performed on stable cell lines cultured on confluent plates following EGF stimulation (100 ng/ml) for 5 min. All plates were washed twice in ice cold PBS with 1 mM sodium orthovanadate and lysed on ice with 0.7% 3-{(cholamidopropyl)-dimethyl-ammonio}-1-propaneosulphonate (CHAPS) lysis buffer (50 mM Tris HCl pH 8.0, 0.7% CHAPS, 50 mM NaCl, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin) or PLCγ lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton X-1, 1% EGTA, 20 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Each lysate was cleared by centrifugation for 15 min at 4°C. Immunoprecipitations were performed by incubating with the appropriate antibodies: anti-Src (LA074, Quality Biotech; v-Src Ab1, Oncogene Science), anti-EGFR (Transduction Labs) or normal mouse serum (NMS) and protein-G-sepharose for 3 h rotating at 4°C and subsequently washed five times with 0.7% CHAPS lysis buffer. Samples were fractionated by SDS–PAGE and transferred onto PVDF membranes.

For immunoblotting the following antibodies were used, anti-phosphotyrosine (PY20 1 : 1000, Transduction Labs); anti-EGFR (E12020 1 : 1000, Transduction Labs); anti-Neu (Ab3 1 : 1000, Oncogene Science); v-Src (Ab1 1 : 1000, Oncogene Science); phospho-MAPK and MAPK (1 : 1000, NEB); phospho-EGFR Y1068 (1 : 1000, NEB); phospho-Src Y416 (1 : 1000, NEB). Direct binding assays have been described previously (Dankort *et al*., 1997).

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c-Src-null mice exhibit defects in normal mammary gland development and ERα signaling

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The c-Src tyrosine kinase has been implicated to play an integral role in modulating growth factor receptor, integrin and steroid receptor function. One class of steroid receptors that c-Src modulates is the estrogen receptor α (ERα). Although there is strong biochemical evidence supporting a role for c-Src in ERα signaling, the consequence of this association is unclear at the biological level. To explore the significance of c-Src in ERα signaling, we studied the development of various reproductive organs that are dependent on ERα in c-Src-deficient mice. We show that the loss of the c-Src tyrosine kinase correlates with defects in ductal development as well as in uterine and ovarian development. Genetic and biochemical analyses of c-Src-deficient mammary epithelial cells also revealed defects in the ability of mammary epithelial cells to activate a number of signaling pathways in response to exogenous estrogen stimulation. Taken together, these studies demonstrate that c-Src plays a role in ERα signaling *in vivo*.
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Keywords: estrogen receptor; c-Src; mammary gland

Introduction

One of the predominant regulators in the promotion of normal mammary epithelial proliferation and breast cancer is 17β-estradiol (E2) and its receptors, estrogen receptor α and β (ERα and β). Significant progress in elucidating the molecular mechanism of estrogen receptor function has been achieved with the detailed characterization of the receptor–ligand interaction as well as identifying the various signaling molecules that associate with the receptor. The biological importance of ERα was demonstrated with the generation of the estrogen receptor α knockout (ERKOα) mouse that display defects in ovarian, uterine and mammary gland development (Lubahn *et al*., 1993; Korach *et al*., 1996). Taken together, these observations suggest that ERα plays a distinct physiological role in estrogen-mediated signaling.

The functions governed by the estrogen receptor in physiological and pathological events have been found to rely on its ability to crosstalk with a variety of other signaling proteins upon its activation. For example, the c-ErbB2 receptor, intimately linked to breast cancer progression, is known to be when overexpressed an indicator of poor patient prognosis that also correlates with the loss of estrogen receptor function (Newman *et al*., 2000) and tamoxifen resistance (Gullick *et al*., 1991; Grunt *et al*., 1995; Carlomagno *et al*., 1996; De Placido *et al*., 1998; Houston *et al*., 1999), suggesting that surface and nuclear receptor crosstalk may promote the conversion to an aggressive tumor phenotype. The modulation of multiple cytoplasmic molecules, such as PI3′K (Simoncini *et al*., 2000), Akt/PKB (Campbell *et al*., 2001; Hisamoto *et al*., 2001), mitogen-activated protein kinase (MAPK) pathways (Migliaccio *et al*., 1993, 1998; Kato *et al*., 1995; Di Domenico *et al*., 1996), and cyclinD1 (Zwijsen *et al*., 1997; Zwijsen *et al*., 1998; Castoria *et al*., 2001), have also been found to be associated with ERα activation. Evidence further demonstrates that signaling via the c-Src protein tyrosine kinase plays an important role in ER activation (Migliaccio *et al*., 1993, 1998, 2000; Di Domenico *et al*., 1996). Specifically, c-Src has been found to directly phosphorylate ERα on Y537, promoting its transactivation (Arnold *et al*., 1995a, b, 1997). Furthermore, ERα signal transduction can be attenuated using Src inhibitors (Migliaccio *et al*., 1996). In addition, c-Src has been found to impinge on DNA proliferation, cell survival pathways and cell cycle progression in response to estrogen (Castoria *et al*., 2001).

Given the mammary gland phenotype of the ERKOα mouse, in conjunction with the role of c-Src in ER activation, the above data suggest that c-Src may play an important role in ERα-mediated mammary gland

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development. To investigate the potential *in vivo* impact on ERα function, we examined whether the development of a number of hormonally dependent tissues was perturbed in c-Src-deficient mice. The results demonstrate that the mammary gland, uterine and ovarian tissues derived from the c-*src*-deficient animals displayed a dramatic developmental delay. In addition, we demonstrate that mammary epithelial cells (MECs) from c-*src*-null mice inefficiently activate a number of signaling pathways in response to estrogen stimulation. Taken together, these observations suggest that c-Src plays a role in modulating the response of a number of tissues to estrogen stimulation.

Results

Requirement for c-Src for normal mammary ductal morphogenesis

Given the potential importance of c-Src in ERα signaling in the mammary epithelium, we investigated whether female mice carrying a disrupted *c-src* gene exhibited any obvious mammary gland defects. To accomplish this, we performed mammary gland whole-mount analyses on virgin female mice between 3 and 10 weeks of age. In contrast to the normal ductal patterning observed in wild-type mice, examination of the c-*src*-deficient strains revealed a dramatic defect in the development of the ductal tree (Figure 1a). Furthermore, the observed ductal branching defect does not appear to be due to the runted nature of the c-*src*-null phenotype since at 6–10 weeks of age c-*src*-null mice are equivalent in size when compared to their wild-type littermates. In addition to the dramatic effect on ductal outgrowth, the number of terminal end buds (TEBs) in the c-*src*-null mouse is significantly less than that found in the wild-type mammary tree even up to 10 weeks (Figure 1a and b). Taken together, these observations suggest that the mammary defect in the c-*src*-deficient animal potentially phenocopies the observed ductal defect in ERKOα strains.

*Uterine and ovarian phenotype revealed in c-*src*-null mice*

In addition to the mammary ductal phenotype, ERα-deficient mice also possess ovarian and uterine defects (Lubahn *et al*., 1993; Schomberg *et al*., 1999). To examine whether c-*src*-deficient mice possess comparable deficiencies in uterine and ovarian development, we performed both gross and histological analyses of these tissues. Analysis of uteri excised from wild-type (Figure 2a and c) and c-*src*-null mice (Figure 2b and d) shows that they are grossly dissimilar at 3 and 6 weeks of age. While the uterus from c-*src*-null mice appears hypoplastic, the epithelial, myometrial and stromal layers, although reduced in size, are intact (Figure 2e and f). However, unlike the ERKOα mice where the uterus remains underdeveloped, c-*src*-null uteri can support embryonic development (unpublished observations) (Soriano *et al*., 1991).

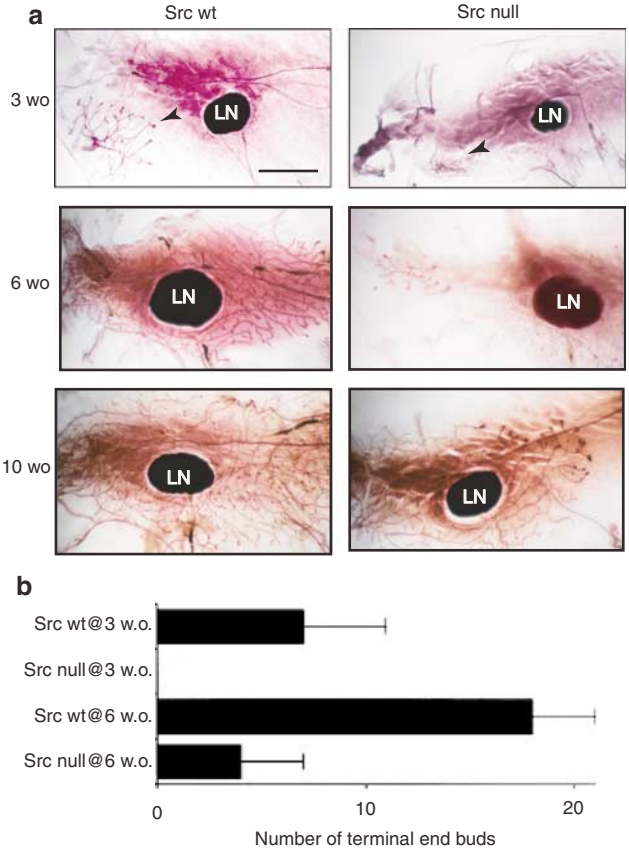


Figure 1 c-Src-null mice display a mammary gland defect. (**a**) Representative whole-mount analysis of 3-, 6- and 10-week-old virgin female mammary glands (fourth inguinal) from c-*src* wild-type or c-*src*-null mice. Arrowhead indicates TEBs. All panels in (**a**) are at × 6.4 magnification. Scale bar, 2 mm (lymph node, LN). (**b**) Number of TEBs counted between wild-type versus c-*src*-null mammary glands are less in c-*src*-null virgin female mice when compared to an age-matched c-*src* wild-type mammary gland ($n = 4$ for each time point)

Histological analysis of ovaries from 4-week-old wild-type mice (Figure 2g and i) displays oocytes at all stages of development from primary oocytes to mature late stage Graafian follicles. In contrast, ovaries from age-matched c-*src*-null mice display only primary and secondary oocytes that do not display any late stage follicular development (Figure 2h and j). However, c-*src*-null mice are fertile, suggesting that there is a general delay in the development of the reproductive system that correlates with the loss of the c-Src PTK. Taken together, these observations suggest that the c-Src-deficient animals exhibit a similar but milder range of developmental abnormalities exhibited by the ERα-deficient strains.

c-Src-null epithelium exhibit defects in their ability to respond to estrogenic stimulation

The above studies suggest that a functional c-Src is required for normal reproductive development, this

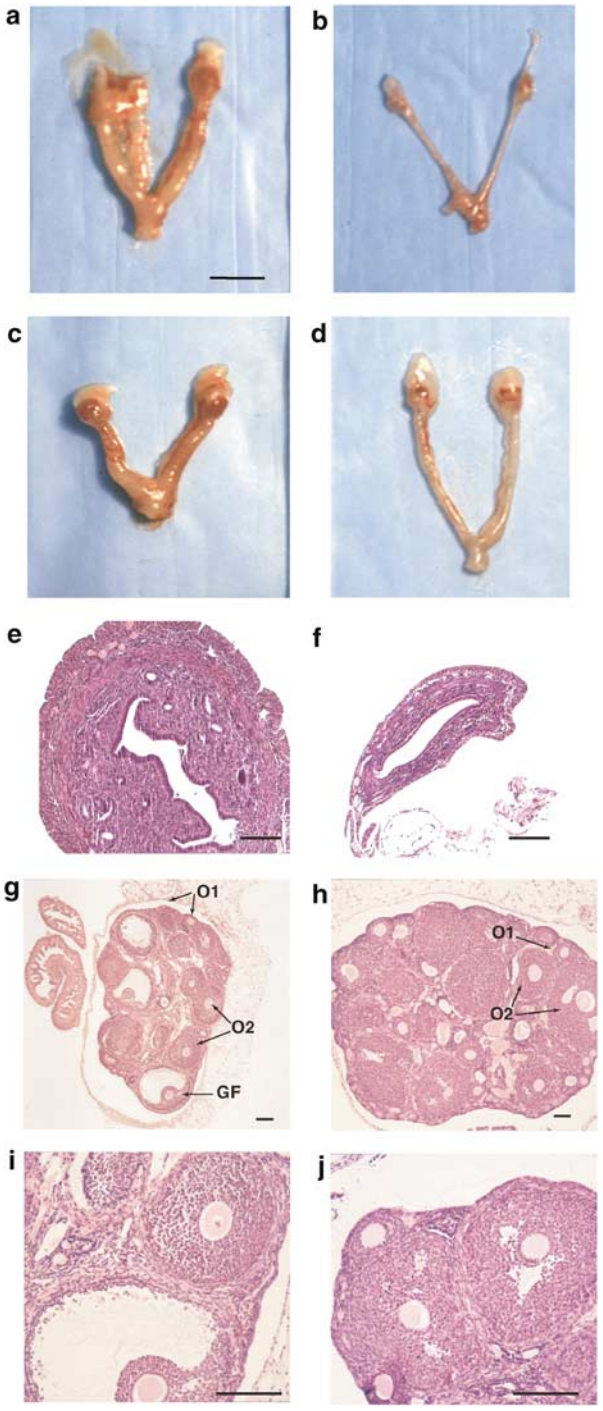


Figure 2 Uterine and ovarian phenotype in c-*src*-null mice. Gross morphology of postnatal uterine organs from wild-type (**a**, **c**) and c-*src*-null (**b**, **d**) mice at 4 and 6 weeks, respectively. Scale bar, 5 mm. Histological examination by hemotoxylin/eosin of a 4-week-old uterus from wild-type (**e**) and c-*src*-null (**f**) mice. Scale bar, 100 μm. Ovaries were excised for histological examination from virgin wild-type (**g**, **i**) and c-*src*-null mice (**h**, **j**) at 4 weeks of age. Scale bar, 100 μm. Primary oocytes (O1), secondary oocytes (O2) and mature Graafian follicles (GF)

being consistent with other studies carried out in tissue culture that suggest that c-Src may play an important role in the MECs capacity to respond to estrogen (Migliaccio *et al*., 1993; Di Domenico *et al*., 1996; Castoria *et al*., 2001). To further address the importance of c-Src in ERα function, MECs from wild-type and c-*src*-null mice were cultivated *in vitro* and their capacity to respond to estrogen stimulation was assessed. Upon estrogen stimulation, wild-type explants display an increase in ERα levels in association with its tyrosine phosphorylation. In contrast, explants derived from the null mammary gland fails to display detectable levels of ER upon estrogen stimulation (Figure 3a and b), these differences not being due to variations in epithelial content as assessed by cytokeratin (Figure 3c). Consistent with the above observations, the loss of c-Src is found to negatively impact basal ER mRNA levels in c-*src*-null MECs (Figure 3d, $*P<0.05$). In addition, MCF-7 cells expressing a dominant negative version of c-Src (Src251) (Kaplan *et al*., 1995) displayed reduced levels of ERα (Figure 3e and f) and tyrosine phosphorylation (Figure 3g) in response to estrogen stimulation. These data suggest that c-Src can modulate the ER at both the transcriptional and translational levels.

An important consequence of the association between c-Src and ERα is the stimulation of the Ras-MAPK signaling pathway (Migliaccio *et al*., 1996; Castoria *et al*., 1999). Indeed, wild-type MECs stimulated with estradiol results in an increase in MAPK activity that peaks at 3 h poststimulation and then decreases in a temporally dependent fashion (Figure 4a–c), while c-*src*-deficient cells peak at 9 h poststimulation. These effects can be attenuated by U0126 (Figure 4d and e), suggesting that in c-Src wild-type and null MECs, estrogen stimulation specifically results in MAPK activation.

The induction of cellular arrest with the use of antiestrogens can be rescued with the overexpression of cyclinD1, suggesting that cyclinD1 plays an important role in estrogen-mediated cell cycle progression (Sicinski *et al*., 1995; Prall *et al*., 1997, 1998). Interestingly, results reveal a lower baseline level of cyclinD1 in the c-*src*-deficient MECs compared to wild type (Figure 4f and g). However, in null MECs, cyclinD1 reaches wild-type levels 9 h poststimulation. No significant difference in mRNA levels of cyclinD1 was detected in c-*src* wild-type versus null samples (Figure 4g), suggesting that the loss of c-Src negatively affects the stability of cyclinD1 in MECs.

Loss of c-Src results in impaired signaling to protein stabilization and survival pathways.

Evidence has suggested that protein stability may play a role in estrogen-mediated signaling (Nawaz *et al*., 1999; Lonard *et al*., 2000). For example, estrogen stimulation results in IRS-1 stability via GSK3β pathway (Morelli *et al*., 2003). To address whether GSK3β activity correlates with our previous data (Figure 3), where ERα levels decrease in the absence of c-Src, MEC lysates were subjected to immunoblot analysis using a phosphospecific (serine 9) GSK3β antibody, its phosphorylation inversely correlating with GSK3β activity leading to an increase in target protein levels (Stambolic and

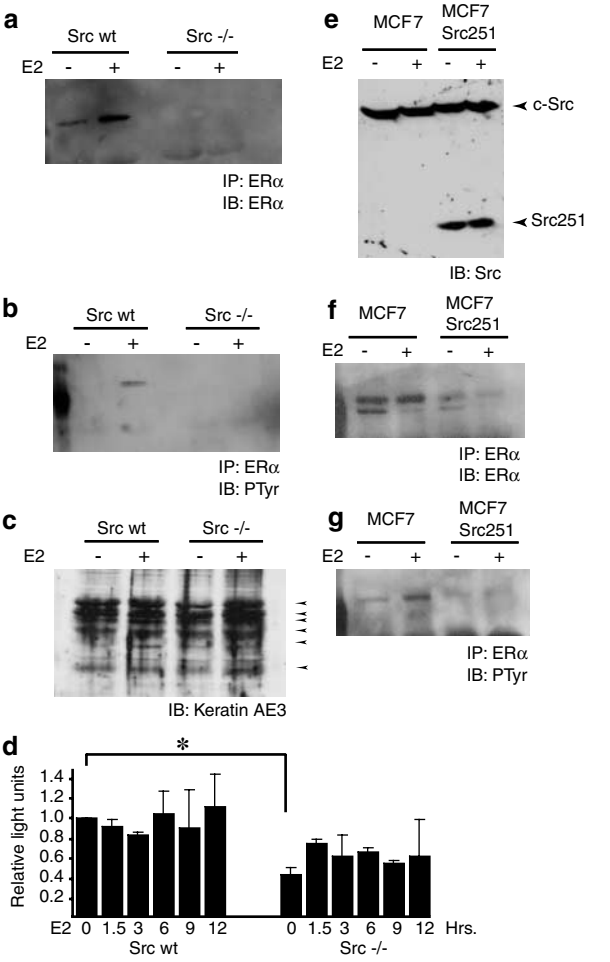


Figure 3 ER phosphorylation is dependent on the presence of wild-type Src. (**a**) Mammary gland explants were cultured in charcoal-stripped serum (CSS) and stimulated with 100 nM of E2 for 10 min. ERα was immunoprecipitated and detected with anti-ERα. (**b**) Additionally, the level of phosphotyrosine was detected by immunoprecipitating ERα and immunoblotting with anti-phosphotyrosine. (**c**) Epithelial content was measured via anti-cytokeratin AE3, recognizing cytokeratins 1, 2, 3, 4, 5, 6 and 8 (67, 65.5, 64, 59, 58, 52.5 kDa). (**d**) Quantitative analysis of ER transcript levels at various time points with E2 was performed on wild-type and c-*src*-null MECs. Represented are two independent runs measured in duplicate and normalized for levels against mPGK. Data are relative to T_0 from c-*src* wild-type MECs. Basal mRNA levels represented as T_0 are significantly different, while time points beyond are not. $^*P<0.05$ (**e**) MCF7 cells that express a dominant negative version of c-Src (Src251) were derived and (**f**) MCF7 and MCF7Src251 MECs were stimulated with 100 nM E2 for 10 min. ERα was immunoprecipitated from each and detected with anti-ERα. (**g**) Additionally, the level of tyrosine phosphorylation was measured by immunoprecipitating with ERα and immunoblotting with anti-PTyr. Results are representative of three experimental runs

Woodgett, 1994; Frame *et al*., 2001) (Figure 5a). Interestingly, c-Src-null MECs display a lower level of GSK3β phosphorylation when compared to wild-type MECs, suggesting that GSK3β activity is greater in c-*src*-null MECs than what is observed in c-*src* wild-type MECs.

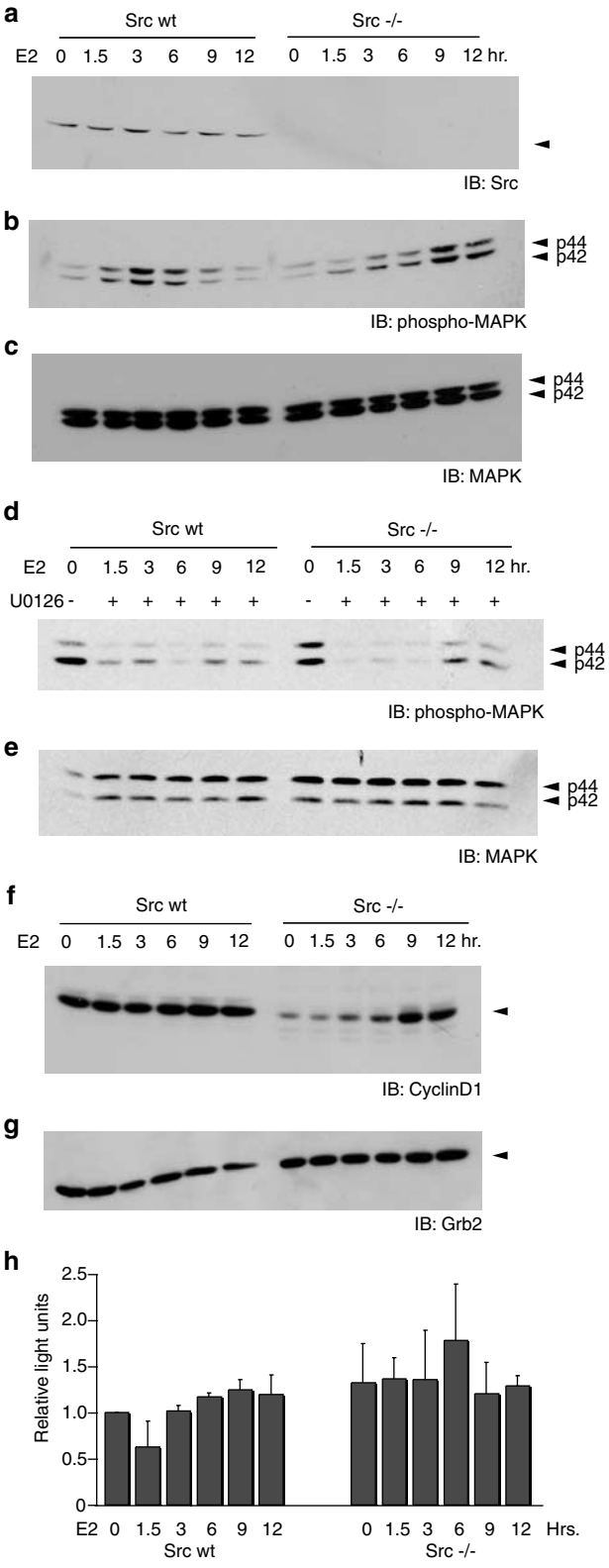
While evidence points to a role for survival pathways in mammary gland development, specifically via Akt (Hutchinson *et al*., 2001), the question of whether c-Src can influence Akt function and thereby modify the mammary gland architecture is unclear. To this end, MECs at various time points stimulated with estrogen were analysed for the activation of Akt (Figure 5b). In the absence of c-Src, we found that Akt activity was suppressed at all time points relative to wild-type upon estrogen stimulation, suggesting that the mammary gland defect may be influenced by machinery associated with apoptosis and cellular survival mediated by Akt.

Discussion

The ability of mammary epithelial cells to respond to and coordinate with a number of different extracellular cues is thought to play a critical role in normal mammary gland development. Here, we demonstrate that germline ablation of c-Src tyrosine kinase has a dramatic defect on the initial stages of mammary ductal outgrowth, uterine and ovarian development. We also show that the defect correlates with a number of molecular pathways that are perturbed in their capacity to respond to estrogen. Whole-mount analyses of the initial stages of mammary gland development revealed a significant delay in mammary ductal outgrowth that was further associated with a decrease in TEB numbers. We also demonstrate that the c-Src-deficient animals exhibit striking delays in both ovarian and uterine development. Of note, ovaries from c-*src*-null mice appear slightly larger than its wild-type counterpart. Interestingly, ovaries from ERKOα mice display a cystic and hemorrhagic phenotype that results in the massive hyperemic ovarian structures (Korach, 1994), somewhat similar to the ovaries derived from the c-*src*-null mouse. However, we believe the similarities end here, given that the c-*src*-null mice do reproduce while the ERKOα mice are infertile. Therefore, compensatory mechanisms may be at work in the c-*src*-null mouse.

In addition to the phenotypic defects in the reproductive organs of c-Src-deficient strains, examination of the capacity of MECs derived from c-Src-null animals to respond to estrogenic stimulation correlate with a requirement for c-Src for an epithelial-specific estrogen response. For example, the loss of c-Src correlates with a decrease in estrogen receptor levels as well as its tyrosine phosphorylation. While it appears that the decrease in estrogen receptor levels work in part at the transcriptional level, a translational and/or post-translational mechanism may also be possible. Specifically, while the transcript levels of ER decrease marginally with the loss of c-Src, the decrease in protein levels appears far more dramatic. Potentially, the signaling events described can be attributed solely on the downregulation of the estrogen receptor by c-Src; however, it is also possible that c-Src plays a more direct role in the signaling pathways investigated which then impact on reproductive function. For example, an increase in GSK3β

activity, as measured by serine 9 phosphorylation, inversely correlates with the levels of c-Src in MECs. The decrease in GSK3β phosphorylation may be due to the decrease in Akt activation in the absence of c-Src.



Given that c-Src can activate PI3′K/Akt (Datta *et al*., 1996; Chan *et al*., 2002) in an estrogen-dependent fashion (Simoncini *et al*., 2000; Campbell *et al*., 2001; Hisamoto *et al*., 2001), which in turn can modulate GSK3β, suggests that an increase in GSK3β activity may potentially impact negatively on the estrogen receptor by initiating its ubiquitination. Indeed, in the presence of estrogen, we see a decrease in ERα levels in MCF7Src251 cells. This suggests that while the SH3 and SH2 domains of Src251 moderately stabilizes ER levels given its presence in nonstimulated MCF7Src251 cells, the kinase region appears necessary for the full stability of the ER, potentially by activating the Akt/GSK3β pathway. Previous reports demonstrate that post-translational events may modulate ER levels,

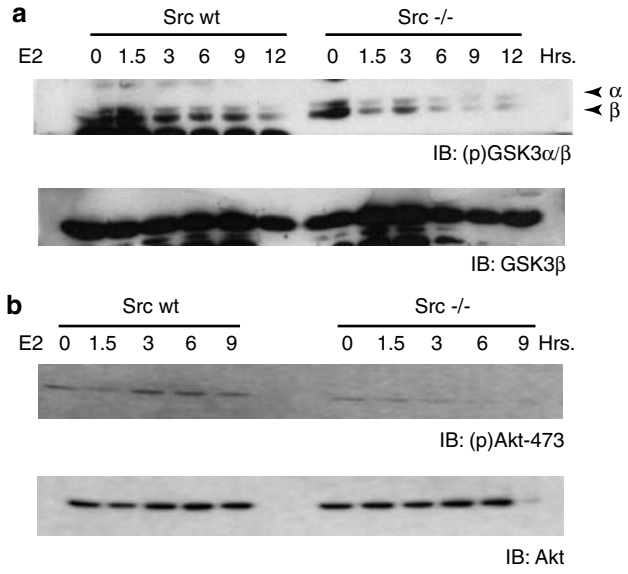


Figure 5 Effects of a loss of c-*src* on GSK3β and Akt function in MECs. Mammary gland explants from wild-type and c-*src*−/− mice were cultivated in CSS, serum starved and exposed to 100 nM E2 over a defined time course. (**a**) GSK3β activity was assessed by immunoblotting with a phospho-GSK3 antibody that detects serine 21 and serine 9 on the α and β forms of the protein, respectively. (**b**) MECs were lysed and probed with anti-Akt serine 473, a phosphorylation site that correlates with the activation of Akt. Data shown are representative of three experimental runs

Figure 4 Absence of c-Src negatively impacts MAPK activation and cyclinD1 in the presence of E2. (**a**) Mammary gland explants from wild-type and c-*src*−/− mice were cultivated, serum starved and exposed to 100 nM E2 over a defined time course. (**b**, **c**) Upon E2 stimulation, lysates were collected and subjected to immunoblot analysis with phospho-MAPK and MAPK. Data shown are representative of three experimental runs. (**d**, **e**) Mammary gland explants from wild-type and c-*src*−/− mice were cultivated, serum starved and exposed to 100 nM E2 either in the absence or presence of 10 μM U0126, an MEK inhibitor, over the total time of stimulation with estrogen and analysed for phospho-MAPK and MAPK. Data shown are representative of two experimental runs. (**f**, **g**) MEC lysates stimulated with estrogen were analysed for cyclinD1 expression with Grb2 as a control and (**h**) quantitative analysis of cyclinD1 transcript levels was performed

specifically, proteosome inhibitors such as MG132 or lactacysin can block ER degradation, suggesting that an ubiquitin–proteosome pathway can mediate the down-regulation of the estrogen receptor (Nawaz *et al*., 1999). Furthermore, estrogen receptor levels have been found to fluctuate in a cyclical fashion on estrogen response element (ERE)-containing promoters via proteasome-mediated degradation initiated by ubiquitination (Reid *et al*., 2002; Reid *et al*., 2003), and inhibition of either the ubiquitination or proteosomal pathways increase estrogen-mediated responses to ERE-containing genes (Fan *et al*., 2004). Interestingly, c-*src*-null mice still retain their reproductive capacity suggesting that there still remains a functional, albeit low, level of ERα that can sustain reproduction in c-*src*-null mice. To this end, it appears that c-Src activity plays a key role in regulating estrogen receptor levels, potentially modulated by protein stability via GSK3β activity.

Given the importance of the c-Src kinase in cellular signaling, the possibility exists that other receptor pathways contribute to the reproductive phenotype, which include c-ErbB2 (Andrechek *et al*., 2004; Jackson-Fisher *et al*., 2004), the fibroblast growth factor receptor (Jackson *et al*., 1997), the β-catenin (Tepera *et al*., 2003) and the Wnt-4/Progesterone receptor pathways (Brisken *et al*., 2000). Therefore, while our data suggest that the estrogen receptor pathway is influenced by c-Src, it would not be surprising that multiple mechanisms likely play a part in the generation of the reproductive phenotype. Further analysis on the role of c-Src on these other pathways in reproductive development would be of great interest.

A significant find in characterizing estrogen receptor crosstalk was its ability to impinge on the Ras/MAPK signaling pathway (Migliaccio *et al*., 1996; Castoria *et al*., 1999). Induction by estrogen leads to the phosphorylation of serine 118 on ERα by MAPK, resulting in ERE binding and transactivation of downstream elements (Joel *et al*., 1995, 1998; Kato *et al*., 1995; Bunone *et al*., 1996). Additionally, c-Src induces ER activation, by phosphorylating tyrosine 537 within the AF-2 region, promoting dimerization (Arnold *et al*., 1995a, b, 1997). This aspect of estrogen receptor phosphorylation is one that is highly contested. For example, some groups have reported the importance of tyrosine phosphorylation on ER (Migliaccio *et al*., 1986; Pietras *et al*., 1995), while others have suggested otherwise (Denton *et al*., 1992; Lahooti *et al*., 1994, 1995; Le Goff *et al*., 1994). Indeed, it appears that conversion of Y537 to phenylalanine results in a minimal effect on ER activation (White *et al*., 1997; Yudt *et al*., 1999). While this conflicts with the role of Y537 in ER activation, as well as the general role of c-Src in mediating this phosphorylation event, it is possible that the phosphorylation of Y537, while not critical in estrogen receptor activation, is however involved in other aspects of receptor function.

Previously, it has been reported that the presence of a dominant negative version of Src can negatively affect estrogen-induced DNA synthesis (Castoria *et al*., 1999) and therefore proliferation. Consistent with this, our observations suggest an inability to maintain basal levels of cyclinD1 in the absence of c-Src. Since there are no significant changes in cyclinD1 mRNA levels, post-translational mechanisms may regulate cyclinD1. Furthermore, this also suggests that the proliferation capacity of c-Src-null MECs may be compromised, similar to other studies (Castoria *et al*., 1999), which taken together suggests that the loss of c-Src influences cell cycle pathways that in turn impinge on normal mammary gland development and potentially on reproductive development in general.

Our work further suggests that cell survival pathways also correlate with the loss of c-Src. Consistent with our observations, it has been previously reported that the p85 subunit of PI3′K, which is a known c-Src substrate (Datta *et al*., 1996; Chan *et al*., 2002), couples to the estrogen receptor pathway within the cytoplasm (Simoncini *et al*., 2000), consequently leading to the activation of Akt (Campbell *et al*., 2001; Hisamoto *et al*., 2001). Additional observations have suggested that a complex exists between a ligand bound estrogen receptor, c-Src and PI3′K resulting in S-phase entry of MCF-7 cells via cyclinD1/cdks upon stimulation with estrogen (Castoria *et al*., 2001), supporting our *in vitro* and *in vivo* data involving the c-*src*-null mammary gland. Of note, the decrease in proliferation and activation of survival pathways in a c-*src*-null background only translates to a developmental delay in the generation of an otherwise functional reproductive system. Nevertheless, these data argue that c-Src may serve as a central node in integrating estrogen-dependent activation of MAPK and PI3′K signaling pathways in reproductive development.

Previous analysis of other signaling molecules further demonstrates that a dynamic interplay exists to control epithelial cell function. For example, EGFR and estrogen receptor function is absolutely necessary in the stroma to induce estrogen-dependent ductal outgrowth (Wiesen *et al*., 1999) (Cunha *et al*., 1997). Currently it is unclear as to which compartment is necessary for c-Src function; however, to address this question, reciprocal transplant analyses of mammary tissues as well as a tissue-specific ablation of c-*src* within the epithelial compartment are currently underway. Estrogenic conditions therefore appear to activate c-Src and MAPKs, resulting in transactivation of ERE-containing genes that can influence epithelial cell growth and development.

Materials and methods

Mouse models, primary tissue cultures and stimulations

The derivation of the c-*src*-null mouse was described previously (Soriano *et al*., 1991). Analysis of all wild-type and c-*src*-null mice, tissues and cell cultures derived from these mice were in the context of an Fvb/n genetic background, backcrossed over 10 generations from its original genetic background (Soriano *et al*., 1991). The MCF7 and the MCF7Src251 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) with penicillin, streptomycin and amphotercin-B. For MEC studies, the whole

inguinal mammary gland of virgin female Fvb/n mice was excised and dissected into small fragments. With the addition of an equal volume of PBS and 2× collagenase/dispase solution (0.05 g collagenase; 0.5 g dispase; 100 ml of PBS), the tissue was incubated at 37°C under constant agitation. The solution was cleared and the pellet was resuspended in DMEM supplemented with 10% FBS/penicillin/streptomycin/amphotercin-B.

To stimulate with estradiol (E2, Calbiochem), cells were incubated with 100 nM of E2 for the required times. Serum starvation of cells consisted of incubation with DMEM supplemented with 0.5% FBS. Stripping of endogenous lactogens was performed as described (Lippman *et al*., 1976; Biswas and Vonderhaar, 1987). For U0126 inhibition (New England Biolabs), cells were exposed to 10 μM of the inhibitor in combination with 100 nM of E2 over the described time course.

Expression constructs and protein analysis

The c-Src251 construct was described previously (Kaplan *et al*., 1995), representing a truncated version of c-Src at amino acid 251. Adherent cells were washed twice in ice-cold 1× PBS supplemented with 1 mM sodium orthovanadate. Cell lysis was in 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Each lysate was cleared by centrifugation and quantitated by Bradford assay as specified by the manufacturer (Biorad). Antibodies used include anti-Src (v-Src Ab1, Oncogene Science), anti-phosphotyrosine (PY20, Transduction Labs), anti-Estrogen receptor (Calbiochem); anti-(p)MAPK and MAPK (New England Biolabs); anti-(p)Akt and Akt (New England Biolabs); anti-(p) GSK3α/β and GSK3β (New England Biolabs); anti-CyclinD1 (Santa Cruz) and anti-cytokeratin AE3 (Biomeda). Immunoprecipitation analysis was performed as previously described (Dankort *et al*., 1997).

RNA analysis and quantitative PCR

Flash-frozen tissue was prepared using the reagent TRIZOL® (GibcoBRL). RNA samples were isolated as per the manufacturer's instructions. Using a fluorescence-based method (LightCycler™, Roche), each reaction was set up under the following conditions: CyclinD1 7 mM $MgCl_2$, 0.5 μM primers, 200ng of RNA; ERα 5 mM $MgCl_2$; 0.3 μM primers, 400 ng RNA; PGK 6 mM $MgCl_2$, 0.5 μM primers. Conditions were optimized for each primer pair such that one specific melting curve was obtained. Quantitation was performed using LightCycler v1.2 software. Murine Estrogen receptor α forward 5′-ACACGTTTCTGTCCAGCACC-3′, reverse 5′-GCCTTTGTTACTCATGTGCC-3′; mCyclinD1 forward 5′-CCCGCTGGCCATGAACTAC-3′, reverse 5′-GTGTGTGCATGCTTGCGG-3′; mPGK forward 5′-CACAGAGGATAAAGTCAGCC-3′, reverse 5′-ATAGACGCCCTCTACAATGC-3′.

Mammary gland analysis

The inguinal gland was prepared as previously described (Webster *et al*., 1995).

Statistics

mRNA levels are standardized against mPGK as an internal control and normalized to T_0. Results are presented as mean$\pm$s.e.m. Student's *t*-test was used to evaluate the statistical significance (defined as $P<0.05$).

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